

Evaluation of Antibacterial and Anthelmintic Activity of Fermented Ayurvedic Formulations

Athira R Nath¹, Vishesh Awasthi¹, Thamara K¹, Deepak NG² and Sowmya Kumar^{1*}

¹Department of Life Science, Mount Carmel College, Autonomous, Bengaluru, Karnataka, India, sowmyang11@gmail.com

²Ayurveda Interdisciplinary Research Minds, Mysore- 19, Karnataka, India

Abstract: Traditional medical care systems are believed to incorporate natural ingredients into potential medicines. Lack of standardization has led Ayurveda as a marginalized medical practice. This study aims to throw some light on selected fermented polyherbal formulations against a rare but fatal infection causing *C. violaceum* and intestinal *E. coli* flora. The process of fermentation transforms the actual phytochemicals into a therapeutic molecule. Alcohol produced as a result of fermentation and the phytochemicals exhibits an antibacterial property. Determination of phytochemicals in the formulations and alcohol content makes bacteria and helminths susceptible to the treatment. Phenols, alkaloids and steroids were found to be positive for all the formulations, i.e., Chandanasava, Punarnavasava and Vidangarishta. Assessment of fermented Ayurvedic formulation reveals that it is a potent vermifuge with the goodness of nature. Among the formulations Vidangarishta was found to be most potent in paralysing worms. The results of the study implicate cytotoxic effects of these formulations against *C. violaceum* and intestinal *E. coli* flora and also their ability to act as an anthelmintic agents.

Index Terms: Anthelmintic, Chandanasava, Punarnavasava, Vidangarishta, polyherbal formulation, *C. violaceum*

I. INTRODUCTION

Ayurveda is a branch of medical sciences which is considered the world's ancient medical care system originated from India. The evidence of such medical practices is written in Vedic texts (Chandra, 2016). India is a country that has a rich heritage of traditional medicine. The research on these traditional systems of medicines will help to preserve the traditional heritage also, promotes the use of traditional medicines in the health industry. Herbal formulations are penned ancient scripts of various countries and sub-continent. According to World Health Organisation, more than 80% of the global population entrusts the use of traditional medical care over the modern health care

system. This is because traditional medicine incorporates herbs and plants for treatment, which is likely to put oneself in the hands of nature (Parasuraman et al, 2014). Use of multiple herbs in a predetermined ratio the therapeutic value of the formulation (product) gets increased. The herbal extracts are mixed and allowed to ferment for a few days helps in enhancing the therapeutic value of the product. The method of preparation of each Ayurvedic formulation is written in *Sarangdhar Samhita*- an Ayurvedic literature (Parasuraman et al, 2014).

Fermented traditional medicines (FTM), are one essential component of the Ayurveda currently. The FTM is generally classified into two major types, arishta i.e. fermented decoction and asava i.e. fermented infusion. These fermented formulations hold multiple therapeutic value. Asavas and aristas are generally produced by the process of fermentation. The fermentation period for an Ayurvedic formulation extends up to a month. Asavas are native fermentations and valuable therapeutics because of their increased shelf-life- which is likely due to the contribution of fermentation (Kumar et al, 2015). Ayurvedic formulations used in the study are *Chandanasava*: Chandanasava (as Chandan=Sandalwood and Asava=Sweetening agent) is one of the known ancient, commonly used Ayurvedic formulations. *Punrnavasava*: Punarnava literally translates into 'something that renews or replenishes the body' i.e., a reviver. *Vidangarishta*: Vidanga arishtam is administered to treat any parasitic and worm infections that cause anemia, gut-related issues and other physical problems. No detailed investigations of antibacterial activity against *C. violaceum*, intestinal *E. coli* flora and anthelmintic activity of the above mentioned has been done. *Eudrilius eugenia* is used for studying anthelmintic activity as it is physiologically and anatomically is same as the helminths (Kirstin Rhys S. Pueblos, 2015). Keeping these lacunae in mind the current study

was initiated to understand the effect of formulations as a potent antibacterial and anthelmintic agent.

II. MATERIALS AND METHODS

A. Procurement of Formulations

The formulations Chandanasava, Vidangarishta, Purnvasava were prepared based on Sharangadhara Samitha by Ayurveda Interdisciplinary Research Minds, Mysore. It was procured from the same for assessing antibacterial and anthelmintic activity.

B. Procurement of Earthworms

Earthworms (*Eudrilus eugeniae*) were hand-picked from a vermicomposting unit of Mount Carmel College, Autonomous, Bengaluru.

C. Procurement of Bacterial strains and culture conditions

Chromobacterium violaceum bacterial cells were procured from National Centre for Microbial Resources, NCCS, Pune. The cultures were provided as Lyophilised ampoules and were sub cultured by inoculating *C.violaceum* onto LB agar and incubated at 30°C for 24 hours. Isolated colonies were sub-cultured and maintained for further studies. *E. coli* culture was procured from National Centre for Microbial Resources, NCCS, Pune sub-cultured and maintained in nutrient agar at 37°C.

D. Phytochemical evaluation of ayurvedic formulations

Understanding the phytochemical constituents of the herbal formulations serves as a method to analyse its medicinal value. Apart from the medical aspect, the compounds can also interfere with other reagents used for analysis. The efficiency or toxicity of a formulation is dependent upon the presence or absence of a particular chemical compound. Therefore, biochemical evaluation of Ayurvedic formulations helps in finding the relationship between chemical and biological action of the drug (Sarojini et al., 2020).

1) Qualitative Analysis of ayurvedic formulations

Tannin test (Gelatin test): 3 ml of sample was treated with 1% of gelatin and 10% solution of Sodium chloride. Presence of white precipitate indicated the presence of tannin (Kharat, 2021).

Test for phenol (Ferric chloride test): 3 ml of sample was allowed to react with 10% Ferric chloride solution. Appearance of blue-purple colour indicated the presence of phenol (Bulgariu et al., 2018).

Test for alkaloid (Wagner's test): To 2 ml of sample, Wagner's reagent (prepared by adding 1% of Iodine to 10% potassium iodide) was added. Appearance of reddish-brown precipitate indicated positive test (Parbuntari et al., 2018).

Test for protein (Biuret test) : 2-3 ml of sample was treated with 4% NaOH and 1% CuSO₄ solutions were added. Appearance

of violet colour indicated the presence of protein (Sigma- Aldrich, 1995).

Test for carbohydrate (Benedict's test): To 3 ml of sample 1 ml of Benedict's reagent was added and heated in boiling water bath for 5-10 minutes. Appearance of reddish- brown precipitate indicated the presence of reducing sugar (Fine, 1935).

Test for steroids (Salkowski's test): To 2 ml of sample equal amount of chloroform and concentrated H₂SO₄ were added. The appearance of red colour indicated the presence of steroids in the sample (Auwal et al., 2014).

Test for flavonoids: 2-3 ml of sample was treated with 2% NaOH to obtain a yellow colour for the indication of presence of flavonoids (Jan et al., 2017).

Test for anthocyanin: A small quantity of sample was treated with concentrated H₂SO₄ to obtain a yellow-orange colour for the confirmation of anthocyanin presence (Firdouse, 2011).

Test for saponin (Foam test): To 5 ml of distilled water, a volume of 1 ml sample was added and shook well. Presence of saponin was confirmed by frothing result (Jan et al., 2017).

Test for quinones: The sample was treated with a little amount of conc. H₂SO₄. Appearance of red colour indicates the presence of quinone compound.

Test for coumarin: A small volume of sample was treated with a few drops of 10% sodium hydroxide and observed for the appearance of yellow colour. Appearance of yellow colour confirmed the presence of coumarin compound (Firdouse, 2011).

Test for glycoside (Keller-Killiani test): A small volume of sample was treated with 1 ml of glacial acetic acid and allowed to cool. To this conc H₂SO₄ was added along the sides and observed for the appearance of reddish- brown ring at the junction of two layers. This confirmed the presence of glycosides.

2) Estimation of total phenols by FC Method

Standard solution of gallic acid (10mg/ml) was used as standard. 0.2 to 1 ml of standards were taken, Ayurvedic formulations (VI= 5µL, PV= 10 µL, CH= 10 µl) were taken and made up to 5 ml using distilled water. A volume of 0.5 ml of FC reagent was added and incubated at room temperature for 5minute. A volume of 1.5 ml of 20% Sodium carbonate solution was added. The tubes were incubated in boiling water bath for 1 hour for blue colour development. The colour intensity was read at 660nm calorimetrically using suitable blank (Debnath et al, 2015).

3) Estimation of alkaloid by titrimetric method

Ayurvedic Formulations of 15 ml were taken and mixed with 15 ml of 2N HCL. These solutions were transferred into a separating funnel and the mixture was vigorously shaken. 10 ml of the mixture was transferred into a conical flask and added 2,3 drops of methyl red indicator. It was titrated against 0.1 N NaOH. The end point indicates a golden yellow colour. The volume of

NaOH consumed was tabulated, and the experiment was repeated to get concordant values (Debnath et al, 2015).

The total amount of alkaloids was calculated by considering following equivalent
1ml 0.1N HCl=0.0162g of alkaloid.

4) Estimation of alcohol content

Potassium dichromate of 0.092g was added to 4ml H₂SO₄ mixed with water in the ratio of 3:1. Prepared dilutions of ethanol from 300 ppm to 6400 ppm using distilled water and prevented the evaporation using aluminum foil. To a volume of 2.5 ml of diluted ethanol solution, 1 ml of acid chromate solution was added and mixed well. A blank was prepared using 2.5 ml of ethanol and 1 ml of distilled water. The tubes were allowed to incubate in boiling water bath for 10 minutes. The tubes were cooled and absorbance was read at 580 nm spectrophotometrically. A graph was plotted at the concentration on the X-axis and OD at Y-axis (Pourkarim et al., 2020)

E. Analysis of physical parameter of ayurvedic formulations

The physical parameter of the Ayurvedic formulations, odour, taste, colour, pH, density and viscosity were examined.

Organoleptic assay: Colour was examined by taking a small volume of sample in a test tube and held it against natural light. Sensory test was performed to obtain the odor as, and palatability test for examining the taste (Nimbekar et al., 2020).

Analysis of pH: A solution's pH value determines its acidity or alkalinity. Using a calibrated (Using buffer of pH-4 and 7) and standardized digital pH meter, acidity or alkalinity of the samples was determined.

Determination of density: A dry specific gravity bottle was taken and obtained its empty weight. Sample solution was poured brimful and closed the lid to obtain its weight. The formula for density calculation was used for calculation purpose.

where, M is the mass of object (g/cm³ or kg/l)

Source: (NASA Official., 2021).

Determination of viscosity: The viscosity of the samples was measured using Ostwald's Viscometer (Nimbekar et al., 2020).

F. Determination of Antibacterial Activity

Antibacterial activity of Ayurvedic formulations serves as a medium to obtain new medical choices against resistant bacterial infections. Antimicrobial screening using the agar well diffusion method and determining the minimum inhibitory concentration of the samples (formulations) serves in identifying the effect of a polyherbal formulation against *C. violaceum* (Gonelimali et al., 2018).

1) Antibacterial assay- Agar Well Diffusion Method

Chromobacterium violaceum, Gram-negative, facultative anaerobic, non-sporing coccobacillus bacterium and *Escherichia coli*, also known as *E. coli*, is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium was chosen to screen the effects of the herbal formulations i.e., Chandanasava, Purnarnavasava and Vidangrishta.

The screening was done via Agar well diffusion method. For Agar well diffusion method, the formulations were tested against the bacteria and a positive control i.e., Ciprofloxacin. A loopful of bacterial culture was taken and inoculated in the nutrient broth, and incubated for 24 hours.

Muller Hinton Agar was prepared and poured into a petri plate, and cooled to solidify. 0.1ml of inoculum was poured into the plate, the culture was spread in the media using a sterilised cotton swab. Well of around 6mm are punched and teased into the solidified media, and the formulations of 30 µL were added into the well each of concentrations 50% and 100%. A standard antibiotic, i.e., Ciprofloxacin of 0.2 mg/ml – was taken as positive control. The inoculated plates were incubated for next 24 hours at 30° C. The zone of inhibition around the disc was observed and its diameter were recorded (Balouiri et al., 2016).

2) Minimum Inhibitory Concentration (MIC)

Prepared overnight culture of *C. violaceum* in Muller Hinton Agar (MHA). A volume of 300µL of MHA was added to all the wells. And 300µL of sample was added to the first well. This sample was serially diluted by taking 300µL from the first well and so on. To this 100µL of overnight inoculum was added and incubated at 30° C for 24 hours and 48 hours. Using microplate reader, the absorbance was taken at 520 nm (Andrews, 2001).

G. Evaluation of Anthelmintic activity

Screening for anthelmintic activity: *Eudrilus eugeniae* has been chosen to work upon due to their physiological and anatomical analogy with the intestine infesting round worms. They were procured from Mount Carmel College, vermicomposting unit (Kumar et al., 2010).

The worms were washed thoroughly with water to get rid of the adhered dirt, followed by measuring their length. The earthworms, approximately of similar length were placed in petri dishes, one being negative control. i.e., in normal saline solution and one as a positive control. i.e., in Albendazole of concentration 2%, 6% and 10%. The others were kept in petri dishes containing each Ayurvedic formulation Chandanasava, Punarnavasava and Vidangarishta of varying concentrations i.e., 2%, 6% and 10%. The time was noted in a dose dependent manner where the earthworms were observed to lose their motility causing paralysis which is confirmed by either shaking them vigorously or slightly

pricking their body with pin, followed by fading colour of their skin causing death. The reference standard drug used here in the experiment is Albendazole, which causes hyperpolarization of muscle by its GABA agonist action. This mechanism leads the

Sl.no	Tests	VI	CH	PV
1	Tannins test	-	-	-
2	Phenol test	+	+	+
3	Alkaloid test	+	+	+
4	Protein test	-	-	-
5	Carbohydrate test	+	+	+
6	Steroid test	+	+	+
7	Flavanoids test	-	-	-
8	Anthocyanin test	-	+	-
9	Saponin test (Foam test)	+	-	-
10	Quinones test	-	+	-
11	Coumarins	-	-	-
12	Glycoside	-	-	-

opening of Cl^- channels that cause relaxation and depresses responsiveness of contractile action of acetylcholine thereby flaccid paralysis occurs (Das et al., 2011).

STATISTICAL ANALYSIS

Statistical analysis of the data was carried out by Student's t-test. All the experiments were carried out in triplicate on at least three different occasions and the mean of replicate values were taken. Values were expressed as mean \pm SD (n=3). Comparisons were made between the control and the treated groups. ***P < 0.001, **P < 0.01, *P < 0.05 and NS – non significant.

III. RESULTS AND DISCUSSION

A. Biochemical evaluation of ayurvedic formulations

Biochemical evaluations of the formulations showed the following results

Tannins test was found to be negative for Vidangarishta (VI), Chandanasava (CH) and Punarnavasava (PV). Although plant products containing antimicrobial activity should possess tannins, here the test is found to be negative. Since, the sample used were in low quantity, a higher quantity or other sophisticated analytical methods like HPLC can give better result. Phenol and alkaloid tests were found to be positive for CH, PV, and VI. Presence of phenol indicates that the formulations are biologically active, meaning that it acts as antioxidant, cytotoxic, anti-diabetic etc., (Dinakaran et al., 2017). Phenols are able to enhance the pancreatic β - cell regeneration, regulates PPAR γ gene expression and enhances GLUT activation (Vinayagam et al., 2015). Phenols

also have an inhibitory action on α - amylase, and strong action on α -glucosidase in the intestine. This lowers the release of glucose molecules, thereby controlling glucose level in Type-II Diabetic patient. Alkaloid is a compound largely distributed throughout the plant kingdom, especially in the medicinal plant species. Rich alkaloid containing herbs and formulation plays a vital role in its pharmacological activity (Kurek, 2019). The presence of protein in the qualitative analysis were negative for all the formulations. Presence of phenolic compounds interacts with protein content of the sample and prevents their exposure to the reaction. Due to this, the proteins may show a negative result (Sęczyk Ł, 2019). The tests indicated the presence of carbohydrate (reducing sugars) in CH, PV and VI. The potential reason for this result can be that, the herbal ingredients and honey are rich in carbohydrates. The sugar molecules exposed to the reaction can be the unused ones during the fermentation process (Ajibola, 2012). Steroid content in CH, PV, and VI was found to be positive. Plants produce different steroids as a function of their pharmacological and biological activity (Sultan, 2016). Presence of plant steroidal compounds suggests the antibacterial, anthelmintic, and cardiotoxic properties of the polyherbal formulation (Patel, 2015). The formulation samples for CH, PV and VI showed negative results for flavonoids. Flavonoids are phenolic compounds are widely distributed in medicinal plants having antioxidant properties and antibacterial effect (Tungmunnithum, 2018). Since the formulations are polyherbal, there can be at least a minute amount of flavonoid presence. On the other hand, the herbal ingredients are prepared into a decoction in the initial step, and this process might impact on the total flavonoid content (Gunathilake et al., 2018). However, using analytical equipment can give more accurate results. Plant cells performing photosynthesis contain flavonoids and it cannot be added due to the microbial action. Anthocyanin was found to be positive in CH only. Though it is a ubiquitous phytochemical. Anthocyanin is a potent antimicrobial agent that has rich pharmacological applications. It is a compound that is vulnerable to the impact of temperature, metal ions, pH and light (Khoo, 2017). The intermolecular and intramolecular stability is affected during certain chemical reactions. The compound is not exposed effectively in certain formulations. Therefore, chromatography and spectroscopy methods are used for precise analysis (Giubertoni, 2020). Saponin was found to be positive only in VI. Saponin is believed to have anti-diabetic effect on the consumer (El Barky, 2017). *E ribes*, the key ingredient of VI also exhibits anti-diabetic and anti-cancer effect as likely as saponins (Salehi, 2019). Quinone's test was negative in all the formulation except CH. Synthesis of Quinones are by oxidative action of phytoestrogens and its related chemical compounds. It is also an active in redox reaction, and produces Reactive oxygen species that cause oxidative stress to genetic materials (Pallavi Sharma, 2012). Coumarins and Glycosides are found to be negative for all the formulations.

Table 1. Qualitative determination of phytochemical constituents in CH, PV and VI.

Note: CH= Chandanasava, PV= Punarnavasava, VI= Vidangarishta, “+” indicates presence and “-” indicates absence.

B. Quantitative Estimation of Phytochemical Constituents

Quantitative estimation of total phenols, total alkaloid and alcoholic contents were evaluated using different protocols.

1) Estimation of phenol

The concentration of phenol in Ayurvedic formulation Chandanasava was found to be 3.13 mg/ml, Punarnavasava was found to be 2.5 mg/ml and the concentration of phenol obtained from Vidangarishta is 22.62 mg/ml.

The phenolic content of all the three formulations is determined through Folin-Ciocalteu reagent test. The reaction mixture contained the Ayurvedic formulation, sodium carbonate and FC reagent and water in prescribed quantities. The absorbance at 660nm was measured. According to the results obtained Vidangarishta contain the highest concentration of phenolic compounds. *Embelia ribes*, commonly known as Vidanga contain phenolic acids like, caffeic acid, vanillic acid, chronogenic acid, cinnamic acid and coumaric acid, which adds to the phenol concentration in Vidangarishta (Akshay R. Yadav, 2020). $A = 0.0099x + 22.626$, $R^2 = 0.9995$ is the linear equation used to calculate the total phenolic content in Vidangarishta, Chandanasava and Punarnavasava based on the calibration curve of gallic acid; where A denotes absorbance and X is the amount of gallic acid in μg .

HPTLC method of quantitative evaluation of Chandanasava has shown 25.12 $\mu\text{g/ml}$ of Gallic acid. Methanolic extracts of the berries of *Santalum album* showed the highest concentration of phenols. Raisins or Draksha is used for the preparation of Chandanasava contain a higher quantity of Phenolic content. The average estimated amount of phenolic content in raisins is 5% juice, 1% in pulp and 62 in seeds. Hence raisins which is one of the main ingredients of Chandanasava provide potent phenolic compounds as well as it is a good source of Gallic acid also.

From literature study it was found that, the total phenolic content of *Boerhavia diffusa* plant can be identified and analysed using, the techniques including, HPLC, PAD, MS/MS. This technique has identified 10 phenol compounds. The major phenolic component present in leaves is quercetin-3-O-Robinoside, Kaempferol-3-O-robinoside, Caffeoyltartaric acid, quercetin, euphalitin-3-O-robinoside. And in root, the phenolic content identified as, caffeoyltartaric acid. The nature of soil and geographical location also plays an important role in the phenolic content in the plant (Kabtni, 2020).

2) Estimation of alkaloids by titrimetric method

The concentration of alkaloid content present in Chandanasava was found to be 11.98 mg/ml, Punarnavasava was found to be 7.45 mg/ml and Vidangarishta was found to be 20.25 mg/ml which being the highest concentration among the three.

Phytochemicals including embelin, volatile oil, fixed oil, resin, tannin, and christembine present in the berries of *Embelia ribes* provide the highest concentration of alkaloid to Vidangarishta. Amla, another major ingredient used for the preparation of Vidangarishta was found to possess the phytochemicals phyllatine, phyllembein and phyllantidine which contribute to its alkaloid content (Swetha Dasaroju, 2014). *Boerhavia diffusa* which is commonly known as Punarnava plant contain about 0.04% of the alkaloid known as Punarnavine and punarvoside, which is an anti-fibrinolytic agent (Agrawal et al., 2011).

The major ingredients of Chandanasava include Dhataki flowers (*Woodfordia fruticosa*), mango tree bark, raisins, beech wood, Padmaka, lodhra etc are rich in alkaloids which provide alkaloids to the formulation. 0.25% loturine, 0.02% collutrin, 0.06% loturidin are the phytochemicals which possess alkaloid content in lodhra. Several constituents, including, oxycanthine, karachine, taximaline, dehydrocaroline etc are present in Padmaka contribute to the alkaloid concentration in Chandanasava.

3) Estimation of alcohol content

The percentage of alcohol in Chandanasava was found to be 7.62%, Punarnavasava was found to be 6.9% and vidangarishta was found to be 10.9%.

All the formulations contain self-generated alcohol because the formulations undergone fermentation process. Sweetening agents like sugar and jaggery used the base for the fermentation process. These constituents which are used in specific quantities act as the source of carbohydrate in the process of preparation. Sugar and jaggery enhances the bacterial growth rate in the fermentation medium. Rate of fermentation depends upon the amount and quality of these fermentable sugars (Chandan Das, 2019). Honey is used in Vidangarishta instead of jaggery, as molasses. The usual period of fermentation is 30 days. *S.cerevisiae* and *S.pombe* are the types of yeast present during the process of fermentation. Yeast combines with six different species of bacteria form the biocatalyst for the biotransformation of phytochemicals present in Chandanasava. Though yeast shows dominance during the fermentation period, it disappears after 25th day. On literature study, it was revealed that 30 compounds disappeared during this process. As a result of fermentation, 32 new more compounds were formed. Biotransformation of phytochemicals are mediated by the microflora and microorganisms acquired during fermentation (Rowland, 2018). Flowers like dhataki, *Woodfordia fruticosa* etc., which are used in the preparation of these formulation ferment naturally producing microorganisms. *Woodfordia* species contain wild species of yeast, which triggers the process of fermentation. The addition of this

flower found susceptible to enzymatic conversion of alcohol (Randive, 2016).

C. Physical Parameters analysis of ayurvedic formulations

The organoleptic assay performed reveals the results shown above, Chandanasava smells Grape wine like, and tastes sweet due to the presence of Grapes as one of its ingredients.

Whereas, sweet smell of the Punarnavasava and bittersweet taste is due to the presence of honey and herbal roots being part of the ingredients list. The strong acidic smell of the Vidangarishta is due to the presence of Indian Gooseberry also called as amla, the presence of citric acid brings the strong acidic smell and along with it the presence of honey brings about the sour and sweet taste in this formulation.

The native fermentation is one major cause that leads to the browning of the formulation, along with herbal odour and sweet-sour taste. Also, the pH keeps on decreasing while the fermentation occurs, reduction of sugar content has been observed while on the other hand formation of acetic acid was seen along with increased ethanol content.

The density and viscosity are such physical parameters which are affected by the solid content present in each formulation. It has been observed that the solid content has drastically reduced in the asavas than in comparison with arishta (Vinothkanna et al., 2014). The viscosity is measured as Milli Pascals. Seconds at 24°C

Table 2. Analysis of Physical parameters for Ayurvedic formulations

Formulations	Odour	Taste	Colour	pH	Density kg/L	Viscosity (milli pascal)
Chandanasava	Grape wine like	Sweet	Golden brown	3.02	1011.6	0.0455
Punarnavasava	Sweet	Bitter-sweet	Brown	2.97	1068	0.0466
Vidangarishta	Strong acidic	Sour and sweet	Dark brown	2.79	1110	0.0620

D. Determination of antibacterial activity

Antibacterial activity of three ayurvedic formulations were

Concentration (%)	Zone of Inhibition for VI (mm)	Zone of Inhibition for CH (mm)	Zone of Inhibition for PV (mm)
50	15.5±0.5	4.66 ±8.02	2 ±3.46
100	19±1	15.33±0.577	9±7.93

examined against *C. violaceum* and *E. coli*.

1) Antibacterial assay- Agar Well Diffusion method

Agar well diffusion method or Kirby-Bauer Disk Diffusion test was performed to check the action antibiotic (taken as controls) and Ayurvedic formulations against bacterial growth. The result varies on numerous factors, including, preparation of media, thickness of agar, diffusion in agar, incubation periods

etc. The measurement of the zone of inhibition around the wells is measured for result (Balouiri, 2016).

Bacterial culture of 50 µL was swabbed onto the MHA plates with punched wells. A volume of 10 µL of 0.2 mg/ml of ciprofloxacin, each formulation of 50% and 100% were added to the wells and incubated overnight. Plates swabbed with *C. violaceum* plates were incubated at 30°C while *E. coli* plates were incubated at 37 °C. Diameter of zone of inhibition for each test against positive control was taken on the next day. The observations were replicated and the results for antibacterial assays were expressed as Mean ± Standard Deviation.

The diameter of zone of inhibition against *C. violaceum* was observed to be higher in 100% concentration of Vidangarishta and moderate in 50% concentration. The former was found to be statistically relevant by 19±1 and the latter with 15.5±0.5. The inhibitory zone for Chandanasava was found to be maximum at 100% treatment (15.33 ± 0.577) and comparatively nil in 50% dilution with statistical data of 4.66 ± 8.02. On the other hand, Punarnavasava exhibited maximum inhibition with 100% treated with 9± 7.93 and moderate effect in 50% 2 ± 3.46. On comparing the results obtained from each of the formulations, it is observed that highest antimicrobial potential is exhibited by Vidangarishta, followed by Punarnavasava and least in Chandanasava. Under microscope, *C. violaceum* is Gram negative with rod shape but different Ayurvedic formulations have varying effect on the bacteria, this can be due to the phytochemical activity, and the effect of biotransformation due to fermentation processes (Hegazy, 2015).

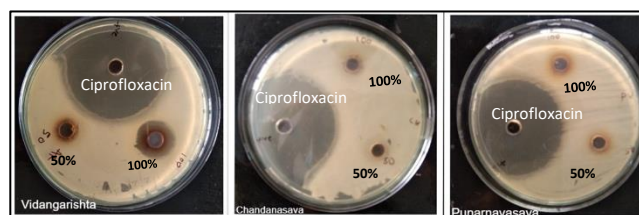


Figure 1: Antibacterial activity of ayurvedic formulations at different concentrations along with positive control against *Chromobacterium violaceum*.

Table 3: Antibacterial activity of ayurvedic formulations at different concentrations against *Chromobacterium violaceum* (Zone of Inhibition expressed as millimetre -mm)

Note: Values are expressed as Mean ±Standard Deviation.

The diameter of zone of inhibition against *E. coli* was observed to be higher in 100% Vidangarishta, moderate in crude sample, i.e., 100% of Punarnavasava and least in crude Chandanasava. Statistical data for 50% of VI was found to be 4 ±4 while crude was found to be 13±1. On the other hand, 4.5 ±4.5 to 50% of PV

and 11.5±1.5 for crude preparation. The Gram characteristics of *E.coli* are negative and appears rod-shaped under microscope .

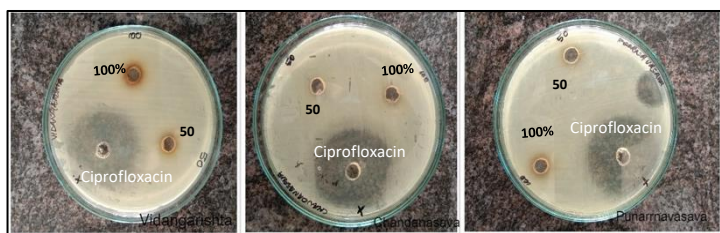


Figure 2. Antibacterial activity of ayurvedic formulations at different concentrations along with positive control against *E.coli* culture.

Table 4: Antibacterial activity of ayurvedic formulations at different concentrations against *E.coli* (Zone of Inhibition expressed as millimetre -mm)

Concentration (%)	Zone of Inhibition for VI (mm)	Zone of Inhibition for PV (mm)
50	4 ±4	4.5 ±4.5
100	13±1	11.5±1.5

Values are expressed as Mean ±Standard Deviation.

However, a certain formulation (including modern medicine) requires a combination of drugs to act effectively against a particular organism. This is because, a combination of phytochemicals broadens the antibacterial spectrum and enhances its property of bacterial growth inhibition. Therefore, it can be inferred that CH might work effectively when it is combined with other formulation against the same organisms (Leekha S, 2011).

2) Minimum Inhibitory Concentration (MIC)

The minimum Inhibitory concentration assay is done in assessing the antibacterial property of the formulations we have chosen. The minimum concentration of the formulation that can inhibit visible growth of bacterium is determined through this assay. The sample was diluted from 100% to the lowest concentration of 0.031%. After incubating the samples for 48 hours with *C. violaceum* culture.

Maximum inhibition of bacterial growth for CH was observed at 6.25% with high statistical significance. *Lodhra* and *P. longum* ingredients along with fermentation exerts maximal antibacterial action in CH (Zou, 2015). *B. diffusa* is a the primary ingredient of PV. Minimum Inhibitory Concentration is the minimum concentration of the antimicrobial agent that reduces the visible growth of microbes after overnight incubation (Andrews, 2001). *B. diffusa* alone has a rich antibacterial property which is enhanced with additive effects of phytochemical constituents of the other ingredients of the formulations (Apu, 2012) . Maximum inhibition of bacterial growth for Vidangarishta was observed at the concentration of 12.5%. With high statistical significance. The secondary metabolites produced by plants, including, flavonoids,

saponins, alkaloids and phenolics can act against bacterial pathogens, which serves the antibacterial activity of the plant thereby the Ayurvedic formulations (El-Anssary). According to the dose given and the type of bacteria embelin exhibit antibacterial property against gram positive and gram-negative bacterium. The MIC result of different concentrations of *E. officianalis* fruit has revealed that, this plant containing bioactive metabolites exhibit a strong antimicrobial activity (Othman, 2019). *E. ribes*, *Holarrhena antidysenterica*, *Pluchea lanceolata*, and *Amla* are some of the ingredients of VI that inhibits bacterial growth.

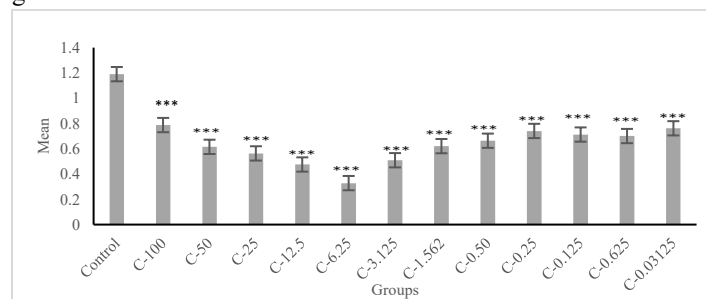


Figure 3. Minimum Inhibitory Concentration of Chandanasava (C-100=100%; to C=0.03125%).

Figure shows the effect of Chandanasava (0.031 to 100 %) on microbial cell growth (at 540nm) of *Chromobacterium violaceum*- MIC. Data represent Mean ± SD of six replicates. Students’s t test, ***P<0.001, **P<0.01, *P<0.05 and NS= Non-Significant. Comparisons were made between treated group versus untreated control.

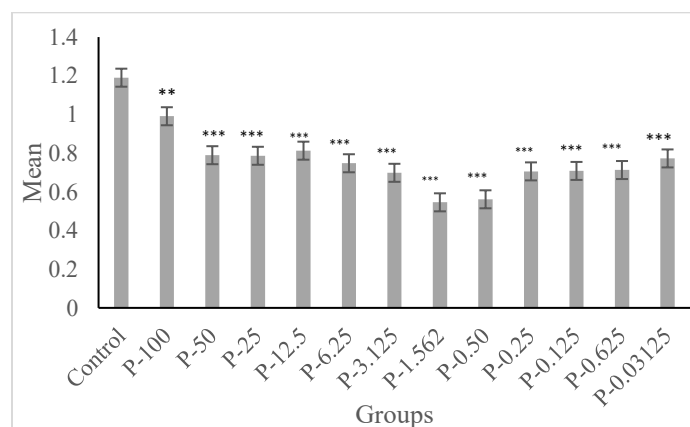


Figure 1. Minimum Inhibitory Concentration of Punarnavasava (P-100% to P-0.03125%).

Figure shows the effect of Punarnavasava (0.031 to 100 %) on microbial cell growth (at 540nm) of *Chromobacterium violaceum*- MIC. Data represent Mean ± SD of six replicates. Students’s t test, ***P<0.001, **P<0.01, *P<0.05 and NS= Non-

Significant. Comparisons were made between treated group versus untreated control.

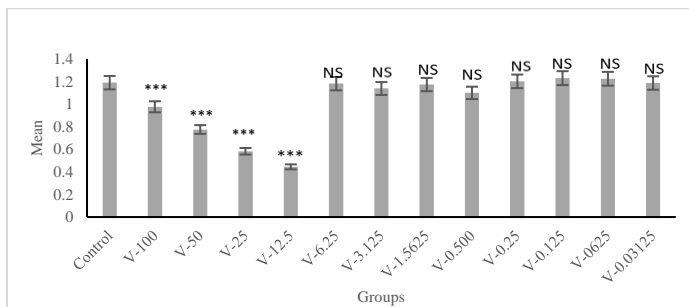


Figure 2. Minimum Inhibitory Concentration of Vidangarishta.(V-100% to V-0.03125%)

Figure shows the effect of Vidangarishta (0.031 to 100 %) on microbial cell growth (at 540nm) of *Chromobacterium violaceum*- MIC. Data represent Mean \pm SD of six replicates. Students' t test, ***P<0.001, **P<0.01, *P <0.05 and NS= Non-Significant. Comparisons were made between treated group versus untreated control.

On comparing the results, PV has the highest capacity to inhibit microbial growth at lowest concentration followed by CH and then VI. MIC for PV was found to be maximum of 1.562% with high statistical significance.

E. Evaluation of anthelmintic activity

The three formulation, i.e., Purarnavasava, Chandanasava and Vidangarishta has successfully shown to affect the worms. The time duration involved to shows the potential. When compared, Vidangarishta has been seen as the most potent, followed by Purnarnavasava, then Chandanasava and Positive control i.e., Albendazole.

It has been found out that the ingredients involved in the preparation of the arishta and asava are a cause of its anthelmintic property. For example, the presence of the Long pepper, Black pepper, Neem etc. The ingredients either alone or in combination shows numerous therapeutic values including anthelmintic properties (Singh S, 2016) .

Table 5. Evaluation of anthelmintic activity of formulations and a positive control (albendazole)

Formulations	2%	6%	10%
Punarnavasava	Paralysed 50 min	Died 67.2 min	Died 16 min
Vidangarishta	Died 67 min	Died 11 min	Died 7 min
Chandanasava	Paralysis 46 minute	36 min	23 min

Positive (Albendazole)	Paralysis 55 min	Paralysed 40 min	Death 60 minute
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Fig 6 Anthelmintic activity of different dilutions of ayurvedic formulation against a positive control (From left to right, Control, 2%, 6% and 10%).

CONCLUSION

Ayurvedic formulations Chandanasava, Vidangarishta, and Purnvasava were found to be rich in the phytochemical components which makes them a potent therapeutic agent. Formulations also showed a promising antimicrobial activity against *C.violaceum* as compared to *E.coli*. They were also found to be a potent anthelmintic agent. Further studies could help in confirming the therapeutic potential of the fermented formulations.

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CONFLICT OF INTEREST

There is no conflict of interest.

REFERENCES

Parasuraman et al. (2014). Polyherbal formulation: Concept of ayurveda. *Pharmacognosy Review*, 8(16), 73- 80.

Kumar et al. . (2015). Phytochemistry And Pharmacology Of Santalum Album L. A Review. *World Journal of Pharmaceutical Research*, 4(10), 1842-76.

Kirstin Rhys S. Pueblos, J. P. (2015). *In Vitro* Anthelmintic Activity Evaluation of the Aerial Part of Ruellia tuberosa Linn. Against Eudrilus eugeniae . *Procedia Chemistry*, 570-577.

Sarojini et al. (2020). Herbal formmulation: Review of efficacy, safety and regulations. *International Journal of Research in Pharmaceutical Sciences*, 11(SPL3), 1506- 1510.

- Kharat, D. A. (2021). *Tannins*. Moshi, Pune: PES Modern College of Pharmacy (for Ladies).
- Bulgariu et al. (2018). Simple and rapid spectrophotometric method for phenol determination in aqueous media. *Bulletin of the Polytechnic Institute of Jassy*, 9- 18.
- Parbuntari et al. (2018). Preliminary Phytochemical Screening (Qualitative Analysis) . *Eksakta* , 1- 8. Retrieved from Sigma- Aldrich. (1995). *Protein Determination- Biuret Method*. Darmstadt, Germany: Sigma Aldrich.Co.
- Fine, J. (1935). Benedict's Qualitative Test-A Further Modification Suitable For Estimation Of Urine Glucose In Ward Or Side Room. *The British Medical Journal*, 1169-1170.
- Auwal et al. (2014). Preliminary phytochemical and elemental analysis of aqueous and fractionated pod extracts of *Acacia nilotica* (Thorn mimosa). *Veterinary Research Forum*, 5(2), 95- 100.
- Jan et al. (2017). Preliminary Phytochemical Screening, Quantitative Analysis of Alkaloids, and Antioxidant Activity of Crude Plant Extracts from *Ephedra intermedia* Indigenous to Balochistan. *The Scientific World Journal* , 1-8.
- Firdouse, S. (2011). Phytochemical investigation of extract of *Amorphophallus campanulatus* tubers. *International Journal of Phytomedicine*, 32- 35.
- Chandra, S. (2016). Ayurvedic research, wellness and consumer rights. *Journal of Ayurveda and Integrative Medicine*, 10(7), 6-10.
- Debnath et al. (2015). Estimation of alkaloids and phenolics of five edible cucurbitaceous plants and their antibacterial activity. *International Journal of Pharmacy and Pharmaceutical Sciences*, 223 -227.
- Pourkarim et al. (2020). A Simple Colorimetric Method for Determination of Ethanol in Exhaled Breath Condensate. *Pharmaceutical Sciences*, 1-10.
- Nimbekar et al. (2020). Formulation and evaluation of fermented. *Intenational Journal of Phytomedicine*, 2(12), 26-29.
- NASA Official. (2021). Measuring Density. *Goddard Space Flight Center : NASA Official*.
- Gonelimali et al. (2018). Antimicrobial Properties and Mechanism of Action of Some Plant Extracts Against Food Pathogens and Spoilage Microorganisms. *Frontiers In Microbiology*, 9, 1-9.
- Balouiri et al. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, 71- 79.
- Andrews, J. M. (2001). Determination of Minimum Inhibitory Concentrations. . *Journal of Antimicrobial Chemotherapy*, 5-16.
- Kumar et al. (2010). Antibacterial and Anthelmintic activity of selected fermented Ayurvedic herbal formulations. *Drug invention today*, 7(2), 347-48.
- Das et al. (2011). Determination of Anthelmintic Activity of the Leaf and Bark Extract of *Tamarindus Indica* Linn. *Indain Journal of Pharmaceutical Sciences*, 11(7), 104-107.
- Siqueira et al. (n.d.). Levels of Tannins and Flavonoids in Medicinal Plants: Evaluating Bioprospecting Strategies. . *Evidence-Based Complementary and Alternative Medicine*, 782- 787.
- Dinakaran et al. (2017). Profiling and determination of phenolic compounds in Indian marketed hepatoprotective polyherbal formulations and their comparative evaluation. *Journal of Ayurvedic and Integrative Medicine*, 1(9), 3- 12.
- Vinayagam et al. (2015). Antidiabetic Effects of Simple Phenolic Acids: A Comprehensive Review. *Phytotherapy Research*, 2(2), 3.
- Kurek, J. (2019). *Introductory Chapter: Alkaloids - Their Importance in Nature and for Human Life*. IntechH open.
- Sęczyk Ł, Ś. M.-D. (2019). Protein-Phenolic Interactions as a Factor Affecting the Physicochemical Properties of White Bean Proteins. *Molecules*, 3(24), 408.
- Das et al. (2017). Physico-chemical Standardization of Asava and Arista Formulations: An Overview. *International Journal of Pharmaceutical Sciences Review and Research*, 193- 201.
- Ajibola, A. C. (2012). Nutraceutical values of natural honey and its contribution to human health and wealth. *Nutrition & metabolism*, 9, 61.
- Sultan, A. (2016). Steroids: A Diverse Class of Secondary Metabolites. . *Medicinal Chemistry*, 5.
- Patel, S. S. (2015). Systemic review of plant steroids as potential anti-inflammatory agents: Current status and future perspectives. *The Journal of Phytopharmacology*, 2(4), 121-125.
- Tungmunnithum, D. T. (2018). Flavonoids and Other Phenolic Compounds from Medicinal Plants for Pharmaceutical and Medical Aspects: An Overview. *Medicines*, 3(5), 93.
- Gunathilake et al. (2018). Effect of Different Cooking Methods on Polyphenols, Carotenoids and Antioxidant Activities of Selected Edible Leaves. *Antioxidants.*, 9(7), 1-12.
- Kumar et al. (2017). Qualitative and Quantitative Analysis of Flavanoids. *Journal of Pharmacy and Biological Sciences*, 1(12), 62-82.
- Khoo, H. E. (2017). Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. *Food & nutrition research*, 1(61), 1361779.
- Giubertoni, G. S. (2020). Effect of intramolecular hydrogen-bond formation on the molecular conformation of amino acids. *Commun Chem* (3), 84.
- El Barky, A. &E.E. (2017). Saponins and their potential role in diabetes mellitus. *Diabetes Manag.* , 7, 148-158.
- Salehi, B. A.A.O. (2019). Antidiabetic Potential of Medicinal Plants and Their Active Components. *Biomolecules*, 9(10), 551.
- Pallavi Sharma, A. B. (2012). "Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions",. *Journal of Botany*, 2, 26.
- Akshay R. Yadav, D. S. (2020). Phytochemical and Pharmacological Review of *Embelia ribes*. *International Journal of Scientific Research in Chemistry*, 5(7), 6.
- Kabtni, S. S. (2020). Influence of climate variation on phenolic composition and antioxidant capacity of *Medicago minima* populations. *Sci Rep* , 10, 8293.
- Swetha Dasaroju, K. M. (2014). Current Trends in the Research of *Emblca officinalis* (Amla). *International journal of pharmacology and science*, 2(24), 150-159.
- Agrawal et al. (2011). *Boerhaavia diffusa* Linn.: A Review on its Phytochemical and Pharmacological Profile. *Asian Journal of Applied Sciences*, 663-684.

- Chandan Das, D. d. (2019). Essential Parameters for preparation of Ayurvedic Fermented Products Asava and Arista: An Overview. *Research journal of Pharmacy and Technology*, 12(3).
- Rowland, I. G. (2018). Gut microbiota functions: metabolism of nutrients and other food components. *European journal of nutrition*, 1(57), 1-24.
- Randive, D. S. (2016). Preparation of Arjunāriṣṭa Using Microbes Isolated from Woodfordia fruticosa Flowers (Dhayati). *Ancient science of life*, 1(36), 42-47.
- Vinothkanna et al. (2014). Tracking the organoleptic and biochemical changes in the ayurvedic polyherbal and native fermented traditional medicines: balarishta and chandanasava. *Int J Pharm Pharm*, 9(6), 521-526.
- Balouiri, M. S. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of pharmaceutical analysis*, 2(6), 71-79.
- Hegazy, M. E.-A. (2015). Microbial biotransformation as a tool for drug development based on natural products from mevalonic acid pathway: A review. *Journal of advanced research*, 6(1), 17-33.
- Leekha S, T. C. (2011). General principles of antimicrobial therapy. *Mayo Clin Proc.*, 86(2), 156-167.
- Zou, L. H. (2015). Antibacterial mechanism and activities of black pepper chloroform extract. *Journal of food science and technology*, 52(12), 8196-8203.
- Apu, A. S. (2012). Phytochemical screening and in vitro bioactivities of the extracts of aerial part of Boerhavia diffusa Linn. *Asian Pacific Journal of Tropical Biomedicine*, 2(9), 673-8.
- El-Anssary, R. A. (n.d.). Plants Secondary Metabolites: The Key Drivers of the Pharmacological Actions of Medicinal Plants. In P. F. Builders, *Herbal Medicine*, intechopen.
- Othman, L. e. (2019). Antimicrobial Activity of Polyphenols and Alkaloids in Middle Eastern Plants. *Frontiers in microbiology*, 10(911).
- Singh S, T. J. (2016). An appraisal of the bioavailability enhancers in Ayurveda in the light of recent pharmacological advances. *Ayu*, 37(1), 3-10.
